



OCR-842.US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of

STEPHEN M. STRITTMATTER

Serial No.: 09/485,601

Filed: May 4, 2000

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: Art Unit 1652  
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: Examiner: K. Kerr  
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For: CENTRAL NERVOUS SYSTEM AXON REGENERATION  
USING RHO PROTEIN INHIBITORS (As Amended)

Declaration Under 37 C.F.R. § 1.132

I, Stephen M. Strittmatter hereby declare as follows:

1. I have an A.B. degree from Harvard and an M.D., Ph.D. from Johns Hopkins. After a residency at Massachusetts General Hospital in Boston, I was a Research Fellow in Neurology there and joined the Neurology Department faculty of Harvard Medical School. After three years, I joined the Yale Medical School faculty in the Neurology and Neurobiology Department and am presently a tenured Associate Professor and have the Vincent Coates Chair in Neurology. I have been engaged in research related to axon physiology, pathology, and regeneration for over a decade. A copy of my C.V. is attached hereto.

2. I am the named inventor of the above-denominated U.S. patent application serial number 09/458,601, which has a U.S. filing date of May 4, 2000, as well as its priority application, U.S. application serial number 60/055,268, filed August 13, 1997, and PCT/US98/16794, filed internationally on August 12, 1998, and published as WO 99/08533 on February 25, 1999, and am therefore well aware of the contents of the application describing central nervous system axon regeneration. I was primary investi-

*Duplicate  
see above  
Considered  
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gator of the research summarized therein and have continued research in this area of neurologic study.

3. I submit this Declaration in connection with issues raised by the Examiner in a Patent Office Action dated August 13, 2001. In it, the Examiner objected to use of the term "rho protein inhibitor" as being overly broad in describing methods of the invention for promoting central nervous system axon growth by administering these inhibitors. The Examiner also stated that my examples using an *in vitro* model would not be not indicative of the *in vivo* results predicted in the application. I disagree. As summarized by the Hall papers cited in the application on page 25, in non-neuronal cells, rho proteins are pivotal regulators of several signalling networks activated by a variety of receptor types, and affect many aspects of cell behavior, including actin cytoskeleton dynamics, transcriptional regulation, cell cycle progression, programmed cell death, transformation, and membrane trafficking. As a GTP-ase, the size of the rho subfamily is small and the molecular size of the proteins are small, making them convenient for study, and so much is known about their properties in different systems, which do not appear to change in *in vitro* or *in vivo* models of the same tissue. Moreover, other rho protein inhibitors are known besides the *C. botulinum* C3 exoenzyme or a C3 chimera like the one described in my application. The C3 exoenzyme is an irreversible inhibitor of rho proteins, so it is convenient to use in inhibition studies and/or for comparison purposes, but, as set out in the application on page 12, panels of compounds can be readily screened for inhibitory activity since assays have been published, and the reagents are commercially available.

4. For example, there is a significant literature on the use of statins (*e.g.*, simvastatin and lovastatin, used for lowering cholesterol) and geranylgeranyltransferase inhibitors (abbreviated GTI or GGTI) as rho protein inhibitors. Rho proteins have a lipophylic isoprenyl group, and these inhibitors prevent the lipid attachment step required for rho protein activation. Maddala, *et al.*, recently reported inhibition of rho and rac GTPases in the presence of lovastatin obtained from Merck and C3 exoenzyme in a human lens epithelial cell line (9). Takemoto, *et al.*, reported rho inhibition in the presence of

simvastatin and GTI-286, a compound available from Calbiochem, and compared inhibition with *C. botulinum* C3 exoenzyme obtained from List Biochemicals *in vitro* in cultured cardiac myocytes and *in vivo* in rat hearts (15). Eberlein, *et al.*, reported inhibition of rho isoprenylation leading to inactive rho using lovastatin, and simvastatin obtained from MSD, Sharp and Dohme as well as GTI-286 obtained from Calbiochem in cultured human renal fibroblasts (4). Lesh, *et al.*, reported inhibition of rho A isoprenylation in cultured human trachea smooth muscle tissue in the presence of inhibitors GGTI-286 and another potent, highly specific inhibitor denoted GGTI-2147 (7). Adnane, *et al.*, described rho inhibition by yet another inhibitor denoted GGTI-298 in a human pancreatic carcinoma cell line (1). Cohen, *et al.*, observed rho inhibition via inhibition of GGTI in a cultured human smooth muscle mammary artery cells by a number of compounds depicted as primarily isoprenyl analogues (though all the structures were not given in the paper because the structures had been described earlier; see ref. 3). Nègre-Aminou, *et al.*, describe inhibition of rho activation by simvastatin obtained by Sankyo in the same kind of smooth muscle model (11). Ohnaka, *et al.*, observed rho inhibition by newly developed statin, pitavastatin (obtained from the Kowa Company in Tokyo) in cultured human osteoblasts rho kinase inhibition by pitavastatin in cultured human osteoblasts (12). All these papers, attached hereto, cite the earlier rho protein literature, and it is against this background that the description of my invention should be read.

5. As set out in my application on page 4, only recently has the contribution of this class of proteins to the regulation of neuronal growth cone motility come under investigation. My finding that rho protein inhibitors such as the C3 exoenzyme used in my application's examples promote central nervous system axon regeneration was first observed in *in vitro* experiments, which most investigators in the field use in initial experiments because they are predictive of *in vivo* physiology. Lehmann, *et al.*, subsequently provided *in vivo* experiments with C3 exoenzyme in central nervous system retinal ganglion cells that confirmed my *in vitro* results, confirming the *in vivo* efficacy of C3 exoenzyme to to promote CNS axon regeneration (6).

6. I have since observed axon regeneration *in vitro* and *in vivo* using another rho protein inhibitor denoted Y-27632. Y-27632 is a stable synthetic compound [(R)-(+)-*trans*-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide di-hydrochloride monohydrate] which has been studied in cultured cells, isolated tissues, and intact animals for some time and is known to inhibit Rho Kinase (abbreviated ROCK; see reference 8). The *in vitro* model I used tests E13 chick DRG neuron growth over various substrata that mimic the environment available for CNS axon regeneration (5). Each of the CNS myelin axon regeneration inhibitors, Nogo-66, Amino-Nogo and MAG-Fc, was tested in a purified protein preparation (Figures 1A-C). As reported in the literature, each of these inhibitory proteins reduces outgrowth in control cultures. After either C3 exoenzyme treatment to inhibit rho protein, or Y-27632 treatment to inhibit the downstream protein ROCK, the outgrowth over the inhibitory substrate is nearly equal to that of untreated cultures on control substrates. The ability of C3 exoenzyme and Y-27632 was also tested with whole CNS myelin preparations (Figure 1D). The ROCK inhibitor Y-27632 (1 M) stimulated outgrowth on high concentrations of myelin to levels near equal to that on control cultures with myelin or Y-27632. Thus, Y-27632 or C3 exoenzyme treatment can reverse the inhibitory effect of CNS myelin on axon outgrowth *in vitro*.

7. The Y-27632 compound has been tested in an *in vivo* model of CNS axon regeneration. Y-27632 or vehicle was delivered to rats intrathecally at the site of a mid-thoracic dorsal hemisection injury via an osmotic minipump. A dose of 0.1 g Y-27632/kg body weight per day was administered for 2 weeks in this protocol. A standardized BBB locomotor score (0-21, with 21 being normal function, ref 2) was employed to assess the degree of functional recovery after dorsal hemisection of the spinal cord with or without Y-27632 treatment (Figure 2). Those animals receiving Y-27632 have significantly higher BBB scores at 14 days after the spinal cord injury.

8. The integrity of the descending corticospinal tract (CST) was traced by biotin-dextran-amine (BDA) injection into the motor cortex. In rats receiving the vehicle control, the prominent dorsal CST is tightly bundled above the lesion site (Figure 3A) and abruptly

stops at the transection site. No fibers are seen in the dorsal cord below the injury site in control animals (Figures 3B, C). In the Y-27632 group, some dorsal CST fibers cross from the rostral to caudal cord. Such dorsal CST fibers are visualized in the lower thoracic spinal cord of the Y27632-treated rats (Figures 3D, E). The regenerating axons are seen as far as 15 mm caudal to the injury site. Thus, inhibition of ROCK *in vivo* can promote axon regeneration and functional recovery after spinal injury in rats.

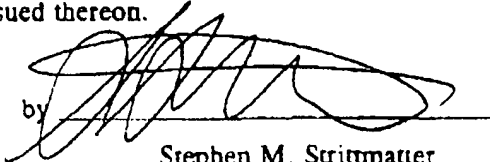
9. Smirnova, *et al.*, have published results showing that lovastatin blocks thrombin-induced neurite retraction, thus promoting neurite outgrowth by blocking isoprenylation of rho in model motoneurons in two cell line cultures (14). Neurite outgrowth using lovastatin inhibition was similar to what was observed using a highly cell-penetrant chimeric molecule consisting of C3 exoenzyme from *C. botulinum* and diphtheria toxin. Sasaki and Takai have described rho inhibition by small soluble proteins called Rho GDIs (13). Linseman, *et al.*, have published studies showing *Clostridium difficile* toxin B inhibits several members of the rho family (8). Västrik, *et al.*, have recently described cell permeant peptide inhibitors of rac and their ability to block the action of certain axon repulsive agents (17).

10. My own results and that reported by others support my claims drawn to the use of rho protein inhibitors to promote axon regeneration. My research describes a new method of using previously described rho protein inhibitors and suggests others to be identified using conventional methodology would be useful for promoting axon regeneration in the treatment of the tragic paralysis and anesthesia observed after spinal cord injury, traumatic brain injury, white matter stroke and the like. Therefore, it is my opinion that the above-mentioned application is an original and significant contribution to the field of axon regeneration.

11. I hereby declare that all statements herein of my own knowledge are true, and that all statements made on my information and belief are believed to be true; further that these statements are made with the knowledge that willful and false statements and the

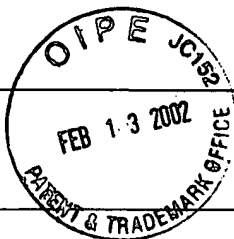
like so made are punishable by fine or imprisonment, or both, under Section 101 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of my application or any patent issued thereon.

Dated: 12 February 2002

by   
Stephen M. Strittmatter

#### REFERENCES

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2. Basso, D.M., *et al.* (1995) *J. Neurotrauma* 12: 1-21.
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4. Eberlein, M., *et al.* (2001) *Brit. J. Pharmacol.* 133: 1172-1180.
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17. Västrik, I., *et al.* (1999) *Curr. Biol.* 9: 991-998.



NAME

**Stephen M. Strittmatter, M.D., Ph.D.**

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
Harvard College, Cambridge, MA	B.A.	1976-80	Biochemistry
Johns Hopkins U., Baltimore, MD	M.D., Ph.D.	1980-86	Medicine, Pharmacology
Massachusetts General Hospital, Boston, MA	Resident	1986-90	Medicine, Neurology
Massachusetts General Hospital, Boston, MA	Fellow	1990-91	Neurology

**NOTE: The Biographical Sketch may not exceed four pages. Items A and B, together, may not exceed two of the four-page limit.**

**Academic Appointments:**

1990-1991 Research Fellow in Neurology, Harvard Medical School  
1991-1992 Instructor in Neurology, Harvard Medical School  
1992-1993 Asst. Professor of Neurology, Harvard Medical School  
1993-1997 Asst. Professor of Neurology and Neurobiology, Yale Medical School  
1997-2000 Assoc. Professor of Neurology and Neurobiology, Yale Medical School  
2000- Assoc. Professor (with tenure) of Neurology and Neurobiology, Yale Medical School  
2000- Vincent Coates Chair in Neurology, Yale Medical School

**Honors and Awards:**

1980 Summa Cum Laude, Harvard College  
1980 Phi Beta Kappa, Harvard College  
1980-1986 N.I.H. Medical Scientist Training Program Awardee at Johns Hopkins  
1985 Michael S. Shanoff Award for research by MD/PhD student at Johns Hopkins  
1991 Certification in Neurology, American Board of Psychiatry and Neurology  
1990-1995 Clinical Investigator Development Awardee, NINDS  
1994-1999 John Merck Scholar in the Biology of Developmental Disorders in Children  
1999-2004 Donaghue Investigator Award

**Peer Review activity:**

1998-2003 Member MDCN-7 Study Section  
Ongoing Manuscript reviewer for Science, Nature, Neuron, Nature Neuroscience, J. Neuroscience, J. Cell Biology, EMBO J., Proc. Natl. Acad. Sci. USA, Development

**Selected reports from 86 articles:**

Strittmatter SM, Valenzuela D, Kennedy TE, Neer EJ, Fishman MC.  $G_0$  is a major growth cone membrane component subject to regulation by GAP-43. *Nature* 1990; 344:836-841.

Cannon SC, Strittmatter SM. Functional expression of sodium channel mutations identified in families with periodic paralysis. *Neuron* 1993; 10: 317-326.

Strittmatter SM, Frankhauser C, Huang PL, Mashimo H, Fishman MC. Neuronal pathfinding is abnormal in mice lacking the neuronal growth cone protein GAP-43. *Cell*, 1995; 80: 445-452.

Goshima Y, Nakamura F, Strittmatter P, Strittmatter SM. Collapsin-induced growth cone collapse mediated by an intracellular protein related to *unc-33*. *Nature*, 1995; 376: 509-514.

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## Research Support

### CURRENT

#### "Axonal growth cone signal transduction"

Principal Investigator: Stephen M. Strittmatter, M.D., Ph.D.

Agency: National Institute of Neurological Disorders and Stroke

Type: R01 (NS 33020, Years 8-12) Period: July 1, 2000 to June 30, 2005

This project explores signal transduction in axonal growth cones of cultured neurons, and is focused entirely on the mechanism of semaphorin action.

#### "Molecular mechanisms of axonal regeneration"

Principal Investigator: Stephen M. Strittmatter, M.D., Ph.D.

Agency: National Institute of Neurological Disorders and Stroke

Type: R01 (NS/HD 39962 Years 1-5) Period: April 1, 2000 to March 28, 2005

This project deals generally with the determinants of axonal regeneration in the adult animal. One aim addresses the role of semaphorins, one aim deals with the role of Nogo in adult CNS injury and one aim utilizes microarray expression profiling to characterize genes regulated by peripheral nerve injury.

#### "Nogo Receptor in Axonal Regeneration"

Principal Investigator: Stephen M. Strittmatter, M.D., Ph.D.

Agency: National Institute of Neurological Disorders and Stroke

Type: R01 (NS 42304, Years 1-5) Period: July 1, 2001 to June 30, 2006

This project seeks to determine the molecular mechanisms of Nogo Receptor action and to determine the role of the Nogo Receptor in the success or failure of adult CNS axon regeneration after injury.

#### "Investigator Award"

Principal Investigator: Stephen M. Strittmatter, M.D., Ph.D.

Agency: Donaghue Foundation

Type: Investigator Award Period: January 1, 1999 to December 31, 2004

This is an Investigator award to support Connecticut research laboratories. Particular projects include the analysis of Go protein knockout mice, the analysis of growth cone endocytosis and the semaphorin action.

#### "Brain and Memory Disorders Award"

Principal Investigator: Stephen M. Strittmatter, M.D., Ph.D.

Agency: McKnight Foundation

Type: Investigator Award Period: April 1, 2001 to March 31, 2004

This award is focused on the potential role of Nogo in adult brain plasticity. The proposed experiments will utilize both Nogo and NogoReceptor knockout mice to study these issues. The studies in the McKnight project include the study of Nogo/NgR transheterozygotes and the study of kainic acid-induced and cortical ablation-induced neural plasticity.

#### "Development of Nogo receptor antagonists"

Principal Investigator: Stephen M. Strittmatter, M.D., Ph.D.

Agency: Biogen Corporation

Type: Sponsored Research Agreement Period: May 1, 2001 to April 30, 2005

This sponsored research project is focused on the identification and optimization of Nogo Receptor antagonists.

#### "Nogo in Mouse Models of Alzheimer's Disease"

Principal Investigator: Stephen M. Strittmatter, M.D., Ph.D.

Agency: Institute for the Study of Aging

Type: Investigator Award Period: September 1, 2001 to August 31, 2003

This project is focused on the potential role of Nogo in Alzheimer's Disease. It will study the effect of Nogo antagonists on form and function in mouse models of Alzheimer's Disease.

### COMPLETED

Project 4 "Nogo Expression And Blockade In Spinal Cord Injury"

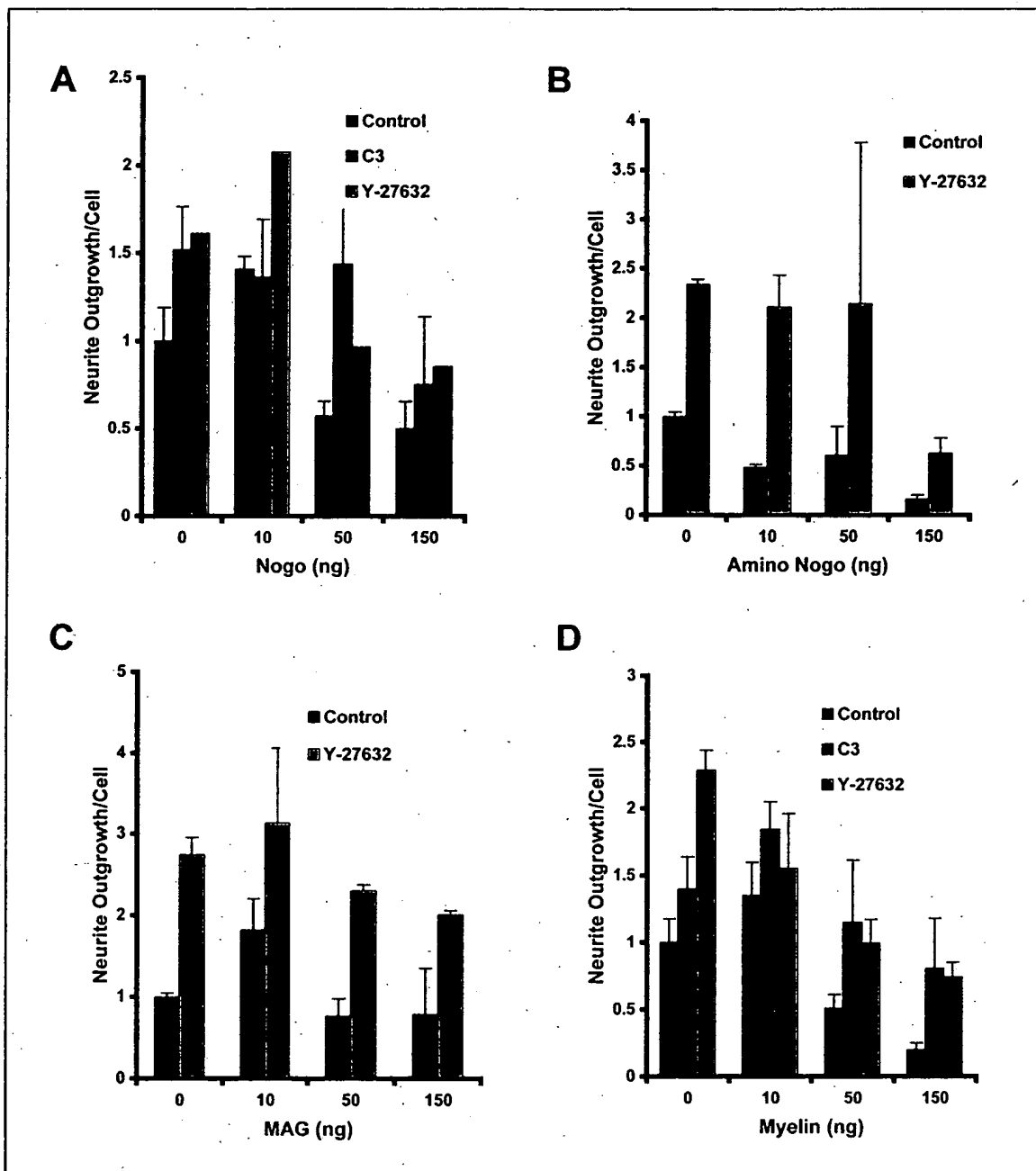
Program Project "Mechanisms of Axonal Plasticity Following Injury"

Principal Investigator of Project 4: Stephen M. Strittmatter, M.D., Ph.D.

Principal Investigator of Program Project: Charles A. Greer, Ph.D.  
Agency: National Institute of Neurological Disorders and Stroke  
Type: P50 (NS 10174, Years 27-29) Period: October 1, 2000 to March 31, 2001

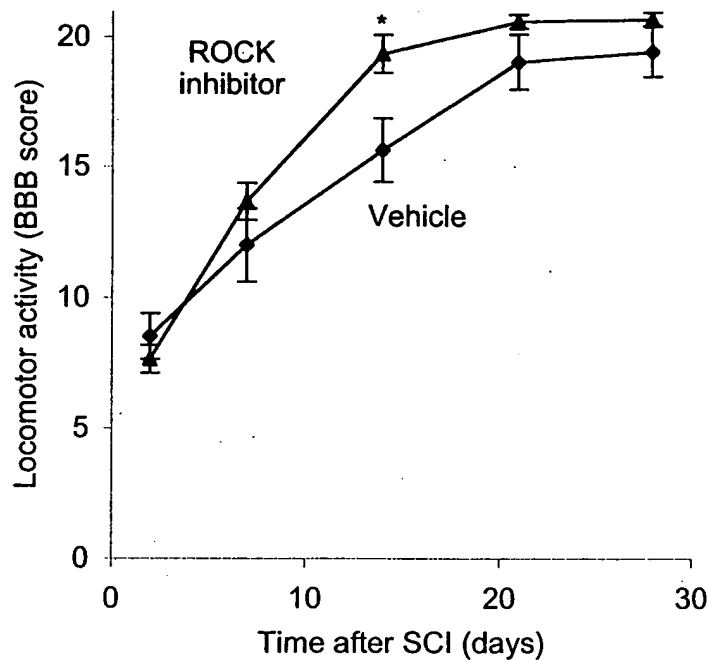
"Rho protein inhibition to promote axonal regeneration"  
Principal Investigator: Stephen M. Strittmatter, M.D., Ph.D.  
Agency: Christopher Reeve Paralysis Foundation  
Type: Research grant Period: January 1, 1999 to December 31, 2000

"Scholar Award"  
Principal Investigator: Stephen M. Strittmatter, M.D., Ph.D.  
Agency: John Merck Fund  
Type: Scholar in the Biology of Developmental Disabilities in Children  
Period: April 1, 1994 to March 31, 1999



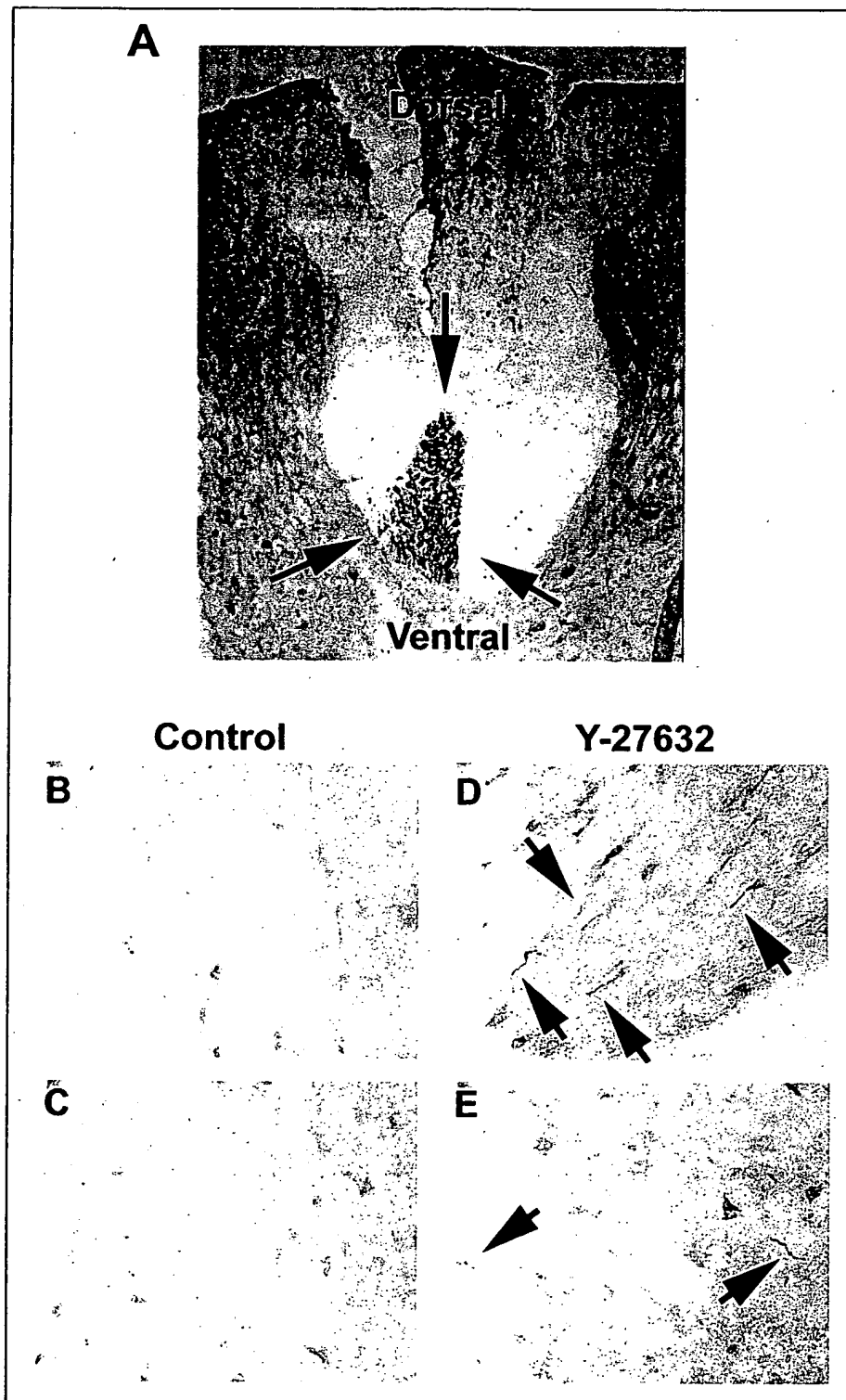
**Figure 1. The effect of C3 exoenzyme trituration or Y-27632 treatment on chick E13 DRG neuron outgrowth.**

Chick E13 DRG neurons were dissociated and plated on different substrates as described previously (ref. 1). As indicated, the cells were tritured with 10 g/ml of purified C3 exoenzyme prior to plating or Y-27632 (1 M) was added to the medium at the time of plating. The data are the means  $\pm$  sem. The substrate coating was purified GST-Nogo-66 in **A**, purified Amino-Nogo in **B**, purified recombinant MAG-Fc in **C** or rat CNS myelin extract in **D**. The nanograms of each substrate protein preparation per 3 l in a dried spot is indicated.



**Figure 2. ROCK inhibitor Y-27632 promotes locomotor recovery after spinal cord injury.** The BBB score (ref. 2) was determined at the indicated times after mid-thoracic dorsal hemisection in vehicle-treated or Y-27632-treated rats. Mean  $\pm$  sem from 10-14 rats in each group. Where indicated, the Y-27632 value is statistically different from control for each day (\*,  $p \leq 0.05$ , Student's *t* test).

**METHODS:** Female Sprague-Dawley rats (weight 175-250gram) were deeply anesthetized with ketamine (60 mg/kg) and xylazine (10 mg/kg). Laminectomy was conducted at spinal levels of T6 and T7, and the spinal cord was exposed. The dorsal half of the spinal cord was cut with a pair of microscissors to sever the dorsal parts of the corticospinal tracts, and the depth of lesion (~1.8 mm) was assured by passing the sharp part of a No. 11 blade across the dorsal half of the cord. An osmotic mini-pump (Alzet ~240 I volume, 0.15 I/hr, 14 day delivery), which was filled with PBS, or 5 mM Y-27632, was sutured to muscles under the skin on the back of the animals. A catheter connected to the outlet of the minipump was inserted into the intrathecal space of the spinal cord at T7 level through a small hole in the dura. Two weeks after lesion, a burr hole was made on each side of the skull overlying the sensorimotor cortex of the lower limbs. The anterograde neuronal tracer biotin dextran amine (BDA, 10% in PBS, 3.5 I/cortex) was applied at 7 injection sites at a depth of 1.5 mm from the cortical surface on each side. Two weeks after BDA injection, the animals were sacrificed by perfusion with PBS, followed by 4% paraformaldehyde. Transverse sections were collected from the spinal cord 11-16 mm rostral to and 11-16 mm caudal to the injury site. The sections were incubated with avidin-biotin-peroxidase complex and the BDA tracer was visualized by nickel-enhanced diaminobenzidine HRP reaction.



**Figure 3 Numerous regenerating CST fibers caudal to a mid-thoracic dorsal hemisection.** **A**, A low magnification view of the spinal cord rostral to a hemisection in a control rat demonstrates the heavy labeling of the dorsal CST fibers. **B-E**, Transverse sections of the dorsal spinal cord white matter at a level 14 mm caudal to the hemisection reveal no CST fibers in sections from two vehicle-treated animals (**B**, **C**) but numerous CST axons in two Y-27632 treated animals (**D**, **E**). Methods as in Fig. 2.

Express Mail Label No.:  
Date of Deposit:

Attorney Docket No.: OCR-842



## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:

APPLICANTS: Strittmatter

SERIAL NUMBER: 09/485,601

EXAMINER: K. Kerr

FILING DATE: May 4, 2000

ART UNIT: 1652

FOR: CENTRAL NERVOUS SYSTEM AXON REGENERATION USING RHO PROTEIN INHIBITORS (AS AMENDED)

#15  
A.G.J  
2/25/02

## DECLARATION UNDER 37 C.F.R. §1.132

I, BERNHARD MUELLER, declare and state that:

1. I am a researcher and I conduct research in the area of the role of rho inhibitors in axon regeneration, which is the subject matter claimed in the above-referenced patent application.
2. I earned my degree in biology at the University of Tuebingen and completed my PhD thesis in Prof. Dr. Friedrich Bonhoeffer's laboratory at the Max Planck Institute (MPI) for Developmental Biology in Tuebingen. After spending one year at Colorado State University (Fort Collins, USA) I returned to the MPI to work on the molecular mechanisms of nerve fiber growth and guidance. In 1996 I became a group leader in the Department for Physical Biology, MPI for Dev. Biology and qualified as a university lecturer in neurobiology in 1999.

During my PhD research I worked on the identification of membrane components that might be attractive or repulsive for growing axons. Thereby I identified, purified, and characterized RGM (Repulsive Guidance Molecule), a new inhibitory protein. Because of its up-regulation in CNS injury and its high axon-growth-inhibitory activity RGM turned out to be one of the most potent nerve regeneration inhibitors. During my postdoctoral fellowship I focused on the role of receptor tyrosine phosphatases and RhoGTPases on axon growth. It is well documented in tissue culture fibroblasts that regulation of the actin cytoskeleton and associated adhesion complexes is controlled by Rho family GTPases (Rho, Rac and Cdc42). I characterized how Rho GTPases and their downstream targets may mediate the motility and collapse of the growth cone as it responds to ephrin-A5, which serve as a paradigm of axon

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inhibitory activity. It is well recognized that mechanisms regulating axon guidance and axon regeneration are likely to be the same. I therefore started to study the role of Rho blockade in CNS regeneration. Recently we found that Rho inhibition was able to suppress all inhibitory activities of the myelin and glial scar. This indicates that Rho and its downstream effector Rho-kinase is the central intracellular signal cascade, and this was the fundament of a series of in vivo experiments that demonstrated the potency of the Rho inhibitor C2C3 to induce nerve regeneration in spinal cord injury.

Since 2000 I am chief executive officer and chief scientific officer of Migragen AG, a company founded by Prof. Dr. Klaus Aktories, Dr. Jan Schwab, Dr. Philippe Monnier and me focusing on nerve regeneration and tumor therapy.

A curriculum vita is attached herewith.

3. Prior to making this Declaration, I was informed by Applicants' representative that the claims in the above-referenced patent application has pending claims generally drawn to a method for promoting central nervous system axon growth in a patient by administration of a rho inhibitor; and that Examiner maintained rejections of the pending claims in the Office Action of August 13, 2001 as lacking enablement. Furthermore, I was informed by Applicants' representative that the Examiner expressed concern that the *in vitro* data presented in the specification have not enabled the scope of the claims since all of the claims require *in vivo* results for enablement. This Declaration presents *in vivo* evidence that administration of a rho inhibitor to an animal with a spinal cord lesion can stimulate nerve growth and restore motor function..
4. I have performed, or have had performed under my supervision, studies directed to demonstrating the ability of rho protein inhibitors to promote axon regeneration in an animal model of spinal cord injury.

#### **Rho inhibitor**

5. C3-transferase was the rho inhibitor used in our studies described herein. A chimeric fusion protein was prepared in order to improve the transport of the active component C3-transferase through the plasma membrane of the cells. The binary actin

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ADP-ribosylating, C2-toxin from *Clostridium botulinum*, was used for this purpose.

C2-toxin is composed of two proteins, the C2I component which is enzymatically active and the C2II component which mediates binding to the plasma membrane and subsequent translocation. The enzymatic activity of the C2I protein is located in the C-terminal region, whereas the binding to C2II is via the N-terminal region. A chimeric fusion protein composed of C3-transferase (from *Clostridium limosum*) and the N-terminal C2I protein was prepared in order to transport the C3-transferase into cells with the aid of this efficient uptake mechanism.

#### **Animals and surgery**

6. 8-12 week old male Lewis rats (220-280 g, Charles River, Sulfeld, GER) were randomly divided into two groups and at least half the spinal cord was severed. One group was perfused with 10 µg C3-C2IN/10 µg C2II. The control animals received either 10 µl C2II alone (without C3 component) or a transection without an injection. Microcapillaries and a stereotactic apparatus were used to inject exactly defined amounts (10x1 µl, 10 µg) of C3-C2IN/C2II toxin into the rostral stump of the severed spinal cord. In order to further stabilize the spinal cord, a device for lifting the rat was constructed which stopped the breathing movement spreading to the vertebral column.
7. After an adequate anaesthesia was achieved, the skin over the spinal column was opened, the muscles adhering to the vertebrae were detached, and a bi-laminectomy was carried out at the level of the thoracic segment TH8. After opening the dura, the dorsal strand of the spinal cord was severed with fine iridectomy scissors in order to carry out a 2/3 overhemisection. The severed neural structures were of motor origin (crossed part of the pyramid pathway, parts of the extrapyramidal pathway) as well as of sensory (dorsal spinal cord) origin. The wound was rinsed with sterile saline and closed.

#### **Sensory and locomotive assessment**

8. Animals were observed over a period of 1-21 days after the injury event using the combined sensory motor Gale score (Gale *et al.*, 1985, *Exp Neurol* 88: 123-134) with incorporation of



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the inclined plane (Rivlin and Tator, 1977, *J. Neurosurg.* 47: 577-581) and the Motor Openfield BBB-score (Basso *et al.*, 1996, *Exp. Neurol* 138: 244-256).

9. In two independent experiments, rats which had received C3-C2IN/C2II exhibited a significant ( $p < 0.0001$ ) improvement of sensor and motor function compared to rats which had only received active or inactive C2-transporter protein or rats which belonged to the control group. The improvement of the sensor and motor function already occurred after the third day and reached a maximum at 21 days after the injury event. Examinations of motor function were carried out such as toe spreading, alignment, straightening up, inclined plane, and of sensory function such as the contraction reflexes of the hind limbs in response to pulling, pain (manual and heat) and pressure as well as swimming tests. A third experiment was carried out as a double blind experimental design and yielded identical results.
10. The improvement of the motor functions of C3-C2IN/C2II-treated animals was shown by the appearance of a functional posture of the hind limbs (usually a bending in the hips, then the knees and then finally a dorsiflexion in the ankles) which reached a maximum (mean  $\pm$  SEM) of 12.2 points ( $\pm 0.84$ ) on the BBB scale (0-21 points) including a supporting of the weight by the hind limbs. In most cases ( $< 80\%$ ), the recovery occurred symmetrically. The control animals reached a maximum of 4.1 points ( $\pm 0.5$ ) on the BBB scale and did not exhibit any progressing improvement during the examined period. On the 10th day after severing, C3-C2IN/C2II-treated animals showed an improvement of up to 8-9 points (sweeping) compared to the first examination time point whereas the control animals were under three points. No significant recovery in motor function occurred in animals which had been treated with C3-C2IN and an inactive C2 transporter component, or which had only been treated with the C2 transporter component or received none of the components (control animals).
11. Sensory recovery was quantified by means of a senso-motor Gale evaluation. Twenty one days after injection, rats treated with C3-C2IN/C2II exhibited a complete retraction of the rear extremities in response to all tested stimuli (touching, mechano-reception, temperature) in a manner that is comparable to untreated rats. C3-C2IN/C2II animals recovered by up to 95% in this combined evaluation, whereas in C2 animals or control animals, recovered was

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less than 50%. In addition, a very pronounced sense of touch was observed in C3-C2IN/C2II animals, which is essential for a specific alignment of the rear extremities.

### **Immunohistological examination**

12. Rats were perfused intracardially with fixative (4% formalin in 0.1 mol/l phosphate buffer, pH 7.5) which contained 20000 IU/l Heparin. The spinal cord and brain were removed and postfixed overnight at 4°C. The fixed tissue was embedded in paraffin, serial sections were prepared, and transferred to silane-coated microscope slides.
13. After fixation with formalin and embedding in paraffin, rehydrated 2 µm pieces were boiled 7 times for 5 minutes in citrate buffer (2.1 g/l sodium citrate, pH 6) and incubated with 10% normal porcine serum in order to suppress non-specific binding of immunoglobulins (Biochrom, Berlin, GER). Antibodies to cell-specific antigens were used to identify particular cell types: glial fibrillary acidic protein (GFAP, 1:100) for astrocytes; myelin basic protein (MBP, 1:200) for oligodendrocytes; Neurofilament (1:200) for neurons. Microglia or macrophages were labelled with monoclonal antibodies to ED1 (1:100), OX-42 (1:100) or ED2 (1:200) using the avidin-biotin-complex (ABC) method in combination with alkaline phosphatase conjugates. In addition, monoclonal antibodies to OX-22 (1:100) were used to identify B-lymphocytes and W3/13 (1:100) to identify T-lymphocytes. OX-6 (1:100) was used to identify MHC-II molecules, in order to characterise the functional immunocompetence. The antibodies were applied to the microscope slides in the solutions stated above containing 1% TRIS-buffered bovine serum albumin (BSA/TBS). Binding was revealed by adding a biotin-coupled second antibody (1:400; 30 min) and an alkaline phosphatase-conjugated ABC complex (1:400 in BSA/TBS; 30 min).
14. The serial tissue sections that were used for immunohistochemistry were stained with Luxol fast blue for myelin. Starting from the centre of the lesion, areas of the tissue were identified at various distances (0.6; 1.2; 1.8; 2.4; 3.0 cm) in a rostral and caudal direction which were obviously damaged or had a deficiency of myelin. The nuclei were identified with cresyl violet (0.1%) in order to differentiate between intact and damaged regions of the grey matter. The sections showed that the rats treated with C3-C2IN in the presence of C2II had less

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secondary damage. The lacunae formation and cavity formation was considerably reduced in the animals treated in this manner compared to the control animals. At the same time more cells, fewer empty spaces and an increased neuron sprouting was found.

15. Morphological changes characterized by (i) reduced scar formation and (ii) reduced secondary injury phenomena such as cavity formation were observed in animals treated with C3-C2IN/C2II. The formation of new tissue was described by immunohistochemical methods (specific antibodies, nuclein staining) and the tissue bridging the site of the lesion was identified as tissue of neuronal origin (neurofilament).
16. A retranssection above the first lesion site (Gh7) led again to paralysis of the hind limbs in the animals which exhibited a regeneration of over 95%. This is due to the fact that the regeneration was in fact due to corticospinal fibers above the first lesion site which bridged the lesion.

#### **Anterograde labelling**

17. 30  $\mu$ l (30  $\mu$ g, 15  $\mu$ g per side biotinylated biodextran (BDA, 10,000 kDa)) was injected by means of a Hamilton syringe into the motor cortex regions. After injection, the wound was washed and closed. This method is used to reveal regenerated axonal fibers of the corticospinal tract (CST). The biotinylated biodextran is transported from the motor cortex regions into the spinal cord. All fibers which contain biotinylated biodextran below the site of the lesion must therefore be the result of new growth.
18. Rats treated with C3-C2IN in the presence of C2II exhibited a considerably increased nerve fiber growth compared to the control animals. The number of fibers as well as the length of the newly grown fibers was considerably increased. The newly grown fibers were GAP43<sup>+</sup> (detected using polyclonal antibodies) and were hence identified as neuronal sprouting fibers.


#### **Summary**

19. In studies directed to assessing the ability of rho inhibitors to promote axon regeneration, we have demonstrated that *in vivo* administration of C3-C2IN/C2II to an animal with a spinal

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cord lesion can stimulate nerve growth, inhibit scar tissue formation, reduce secondary damage, and restore motor function.

20. From our studies, I have concluded that rho inhibitors, such as C3 transferase, when administered *in vivo*, can stimulate axon regeneration and restore motor function.
21. I further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that willful false statements may jeopardize the validity of this application and any patent issuing therefrom.

  
Dr. Bernhard Mueller

Signed at Tübingen, GERMANY  
this 23 day of February \_\_, 2002

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## CURRICULUM VITAE

Name: Bernhard Klaus Mueller  
Birth: October 13, 1959, in Sigmaringen, Germany  
Citizenship: German

## EDUCATION

High School Graduation	Hohenzollern Gymnasium Sigmaringen	1979
Diploma Thesis	University of Tübingen Faculty of Biology Tübingen, Germany	1988
Doctor of Science	University of Tübingen Faculty of Biology Tübingen, Germany	1992

## RESEARCH EXPERIENCE

## Diploma student

April 1987 - September 1988, Max-Planck-Institute for Developmental Biology, Tübingen  
*Advisor: Prof. Friedrich Bonhoeffer*  
*Project: Time-lapse recordings of retinal axons growing on striped membrane carpets.*

## Predoctoral fellow

September 1988 - July 1992, Max-Planck-Institute for Developmental Biology, Tübingen  
*Advisor: Prof. Friedrich Bonhoeffer*  
*Project: A monoclonal antibody recognizes a putative axonal guidance molecule.*

## Postdoctoral fellow

August 1992 - July 1993, Alexander-von-Humboldt Fellowship (F. Lynen), Colorado State University, Department of Anatomy and Neurobiology, Fort Collins, USA

*Advisor: Prof. Stanley B. Kater*

*Project: Transfected parvalbumin alters calcium homeostasis in teratocarcinoma PCC7 cells.*

**Postdoctoral fellow**

August 1993 – March 1996, Max-Planck-Institute for Developmental Biology, Tübingen, Laboratory of Prof. F. Bonhoeffer

*Project 1: Chromophore-assisted laser inactivation of a repulsive guidance molecule (RGM).*

*Project 2: Purification and cloning of the repulsive guidance molecule (RGM).*

*Project 3: Role of fork head genes in positional specification of the chick eye.*

**Group leader**

March 1996 – October 2000, Max-Planck-Institute for Developmental Biology, Tübingen, Department I (Physical Biology)

*Project I: Purification, cloning and functional analysis of the repulsive guidance molecule (RGM).*

*Project II: RhoGTPases in retinal axon guidance*

*Project III: Function of Receptor Tyrosine Phosphatases during formation of the retinotectal projection*

*Project IV: Molecular comparison of invasive tumor cells with invasive neuronal growth cones*

**Collaborations**

Dr. Klaus Aktories, University of Freiburg (Project II)

Dr. Jens Andersen, Odense/Dk (Projects I and III)

Dr. Holger Barth, University of Freiburg (Project II)

Dr. John Bixby, University of Miami (Project III)

Dr. Matthias Mann, Odense/Dk (Projects I and III)

Dr. Andy Stoker, Institute of Child Health London/UCL (Project III)

Member of the DFG funded SFB 430: Cellular mechanisms of sensory processing and neuronal interactions.

January 1997 – December 2002,

Member of the EU Network: The role of protein tyrosine phosphatases in the development and repair of the vertebrate nervous system.  
2000 – 2004

Habilitation in Neurobiology

January 1999, University of Tübingen, Faculty of Biology

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